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Folding aggregated proteins into functionally active forms

Wieslaw Swietnicki

The successful expression and purification of proteins in an active form is essential for structural and biochemical studies. With rapid advances in genome sequencing and high-throughput structural biology, an increasing number of proteins are being identified as potential drug targets but are difficult to obtain in a form suitable for structural or biochemical studies. Although prokaryotic recombinant expression systems are often used, proteins obtained in this way are typically found to be insoluble. Several experimental approaches have therefore been developed to refold these aggregated proteins into a biologically active form, often suitable for structural studies. The major refolding strategies adopt one of two approaches — chromatographic methods or refolding in free solution — and both routes have been successfully used to refold a range of proteins. Future advances are likely to involve the development of automated approaches for protein refolding and purification.

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The current rapid expansion in genome sequencing, protein structure/function prediction, and systems biology offers the possibility that the search for therapeutics or prophylactics against a given target might be performed on a computer instead of in a laboratory. Nevertheless, the final step in the search, a confirmation, will still involve classic laboratory work.

To facilitate the rapid screening of potential protein targets, recombinant expression systems have been developed specifically for the fast, high-yield production of soluble proteins. In general, these expression systems can be divided into three groups on the basis of the host used: bacterial, insect or yeast, and mammalian. Plant systems are not widely used commercially and will not be discussed in the current review.

Mammalian systems offer, in general, human-like post-translational modifications and most proteins produced in this way are correctly folded; unfortunately, however, these systems have the lowest yield. Another problem associated with the mammalian systems is the heterogeneity of the recombinant proteins, owing to post-translational modifications, and the high cost of production [1].

Yeast or insect cells typically provide faster and cheaper systems for protein production, and can offer a higher yield than mammalian systems. However, the protein folding control machinery is less well advanced than in mammalian systems and post-translational modifications, mostly glycosylation, are not as complex [2,3]. This problem is currently being addressed through the use of genetically engineered host cells to give a human-like glycosylation pattern [4].

Bacterial expression systems are the cheapest and fastest, but have two major problems. The protein folding machinery is the least complex and the post-translational modifications of expressed proteins (e.g. glycosylation) are not present. As a result, these systems are generally unsuitable for the expression of glycoproteins and the recombinant proteins produced are frequently insoluble, forming aggregates termed inclusion bodies. Nevertheless, bacterial expression systems are still the most commonly used and, for this reason, the current review will focus on approaches to correct the misfolding of proteins obtained from these hosts.

The current methodologies for refolding aggregated proteins can be divided into two major procedures: chromatographic and non-chromatographic. The first is more expensive, but typically yields a better quality protein, whereas the second is the method of choice for rapid refolding screens.

Chromatographic procedures

Li *et al.* [5] provide a recent overview of chromatographic procedures together with a discussion of the basic concepts. In general, there are three major chromatographic procedures for refolding proteins from inclusion bodies: solvent exchange by size-exclusion chromatography (SEC); on-column refolding (the reversible immobilization of denatured proteins to a solid support and slow removal of the denaturant by solvent exchange); and chaperone-assisted refolding (in which a chaperone is immobilized to a solid support and the partially denatured protein passed over the support to facilitate folding, or vice versa).

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14. ABSTRACT

The successful expression and purification of proteins in an active form is essential for structural and biochemical studies. With rapid advances in genome sequencing and high-throughput structural biology, an increasing number of proteins are being identified as potential drug targets but are difficult to obtain in a form suitable for structural or biochemical studies. Although prokaryotic recombinant expression systems are often used, proteins obtained in this way are typically found to be insoluble. Several experimental approaches have therefore been developed to refold these aggregated proteins into a biologically active form, often suitable for structural studies. The major refolding strategies adopt one of two approaches - chromatographic methods or refolding in free solution - and both routes have been successfully used to refold a range of proteins. Future advances are likely to involve the development of automated approaches for protein refolding and purification.

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Solvent exchange by size-exclusion chromatography

The concept of solvent exchange by SEC is based on the fact that partially denatured protein usually has a high tendency to aggregate when free in solution, owing to the exposure of buried hydrophobic residues. The gel matrix used for SEC restricts the aggregation by physically isolating the molecules, similar to a 'cage' effect, and frequently allows for the successful recovery of biologically active protein. In a typical procedure, isolated inclusion bodies are solubilized in denaturant (e.g. guanidine hydrochloride or urea) and loaded onto a column equilibrated either with the denaturant or with the final refolding buffer. Denaturant-free buffer is then used to elute the protein. Using this approach, Neely *et al.* [6] successfully refolded the β_{1b} subunit of a calcium channel in an active form. In this case, inclusion bodies were first isolated by cell disruption and extensively washed with B-PER mild detergent (Pierce, Rockford, IL) and the purified inclusion bodies resuspended in buffer comprising 6 M guanidine hydrochloride, 0.1 M NaH_2PO_4, 10 mM Tris base, 10 mM dithiothreitol (DTT) (pH 8.0). The sample was then heated to 95 °C to reduce the disulfide bonds, cooled down, and loaded onto a Superdex 200 26/60 preparatory column. The refolded protein was monomeric and tested positively for biological activity in a *Xenopus* oocyte assay by affecting channel conductivity. The refolded protein was also able to bind to a recombinant fragment of the α_1 subunit of the calcium channel. In this example, SEC refolding was the method of choice as attempts to refold the denatured protein by rapid dilution or dialysis were unsuccessful. In another example, Ouellette *et al.* [7] used purified inclusion bodies of human interleukin (IL)-7, which were first denatured and reduced and then loaded onto a Superdex 200 column. The protein concentration was maintained at 0.1 mg/ml and 0.5 M arginine was added to prevent aggregation. After the initial refolding, the protein was further purified by hydrophobic interaction chromatography, ion exchange, and finally SEC. The activity was tested in a cell proliferation assay and was found to be indistinguishable from that of the commercially available protein. In a similar way, SEC refolding was successfully used to obtain biologically active forms of the α5 subunit of the 20S proteasome from human [8], *Pseudomonas fluorescens* lipase [9], ESAT-6 (6 kDa early secretory antigenic target) secretory protein from *Mycobacterium tuberculosis* [10], urokinase plasminogen activator fragment [11], and platelet-derived growth factor [12], among others. In a variation of the scheme, the seven-disulfide bothropstoxin-1 phospholipase was first denatured, reduced and derivatized with 2-nitro-5-thiobenzoic acid to protect the cysteine residues [13]. The protein was then refolded by SEC and the disulfide bonds restored by incubation in a reduced/oxidized glutathione solution. The final activity of the phospholipase was verified using an assay in which the enzyme released a fluorescent dye from artificial liposomes.

On-column refolding

The concept of on-column refolding is similar to that described for SEC refolding, but the protein is reversibly immobilized to a solid support to prevent aggregation during denaturant removal. Depending on the nature of the linkage, the strategy allows for the exploitation of more diverse conditions (e.g. refolding into a non-physiological buffer) and can be used to obtain proteins that are normally degraded or unstable in host cells. The purity of proteins obtained in this way is higher (80–90%) than in typical affinity-capture chromatography, and the proteins are typically free of proteases. The most common strategy uses a six to ten histidine residue affinity tag at the end of the protein for reversible binding to a resin pre-charged with metal, typically nickel. The protein–nickel bond is very stable in the pH range 6.4–8.5 and is usually resistant to denaturants (6 M guanidine hydrochloride, 8 M urea), non-ionic detergents (1–2%), high salt (<1 M NaCl) and low (5–10 mM) concentrations of reducing agents such as DTT, reduced glutathione or phosphine derivatives like Tris(2-carboxyethyl)phosphine (TCEP). Proteins are typically bound to the resin in the denatured state and then slowly refolded by gradually removing the denaturant. To prevent aggregation for aggregation-prone proteins, the resin binding is done in batch mode, typically at concentrations of 1 mg of protein per ml of resin. The refolded protein is eluted with high (100–500 mM) concentrations of imidazole. Oligomeric proteins are typically released using a combination of imidazole and/or pH (pH 5.8 or lower). Because the linkage is very stable, variations of the scheme can be used to form disulfide bridges (typically by using the glutathione/reduced glutathione mix only in the denaturant), to refold proteins into membranes (by including detergent in the refolding mix) or simply to elute a reduced protein (using TCEP or DTT). The disadvantages of the method are the requirement for 300–500 mM salt in the buffer to screen for non-specific binding to the resin and the limited pH range for protein refolding; the former may be a problem when refolding oligomeric proteins sensitive to salt concentrations, and the latter could be problematic when dealing with proteins that are stable under acidic conditions.

In a variation of the on-column refolding procedure, an ion-exchange resin has been used to capture the protein denatured in urea under low salt conditions. The protein is then refolded by the gradual removal of urea and eluted with salt. The method is usually limited to room temperature, as concentrated urea crystallizes easily at 4 °C.

In a typical example of the refolding procedure, on-column refolding has been successfully used to refold human prion proteins of suitable quality for structural studies [14,15]. In this case, total protein from bacterial cells was denatured in 6M guanidine hydrochloride in the presence of 10 mM reduced glutathione (GSH), 100 mM

potassium phosphate (pH 8.0). The protein was then bound to a Ni-agarose resin in batch mode at 4 °C, and the denaturant and GSH removed over a 2 h period. To prevent oxidation, the denaturant with GSH was freshly made and kept on ice during the refolding procedure. In a typical refolding procedure, 20–40 mg of purified refolded protein was obtained per liter of bacterial cell culture in a single refolding step. Including a second refolding step increased the yield by about 50%. Because there was no known interaction partner for the prion protein at that time, the refolded protein was tested for using a monoclonal antibody specific for prion protein. Also, the refolded protein had a tendency to aggregate in a form resistant to proteinase K digestion, as do pathogenic forms of tissue-isolated prion protein. Similar on-column refolding procedures have been used to obtain a whole host of proteins, including enzymes, receptors, antibody fragments and other proteins of immunological significance, among others [16,17•,18••,19–23,24•,25–30]. In an interesting variation of the scheme, denatured inclusion bodies can be bound to an affinity Ni-agarose resin and refolding accomplished by several washes with final buffer containing detergent [31••]. The detergent is then removed by washes with cyclodextrin. Because the procedure can be carried out on gravity-flow columns, it can be easily automated to screen proteins for crystallization.

Chaperone-assisted refolding

The strategy of chaperone-assisted refolding aims to mimic the function of the natural GroEL–GroES chaperonin. *In vivo*, GroEL forms part of a tunnel through which newly synthesized proteins pass to help them form a native structure [32]. GroEL apparently first captures the non-native protein substrate by binding to exposed hydrophobic surfaces, which prevents both aggregation and proper folding of the protein substrate. In a second step, GroEL interacts with ATP and the protein substrate is released in the folded form. GroES also plays a role in the release of the protein substrate. Based on this scheme, there are two methods of chaperone-assisted refolding: one involving the passage of partially denatured substrate through a column that contains immobilized protein chaperone, and the second involving the passage of removable chemicals, 'artificial chaperones', to prevent the aggregation of immobilized protein substrate after elution.

True chaperone-assisted refolding

Most true chaperone-assisted refolding is performed in solution (discussed below); however, a variation of this approach has been developed using chromatography. A denatured protein, human interferon-γ, was passed through a short Sephadex G 200 gel-filtration column to partially remove denaturant and then passed through a second column containing an immobilized GroEL chaperone fragment (amino acids 191–345) [33]. The approach allowed for reuse of the same column and the use of the gel matrix generally prevented aggregation of the denatured protein, similar to the SEC approach discussed earlier. Including the chaperone column increased the refolding yield sixfold, as compared with SEC-only refolding. In another interesting approach, a so-called 'mini-chaperone' GroEL fragment (amino acids 191–345), disulfide isomerase DsbA, and peptidyl prolyl isomerase were all immobilized on an agarose gel and applied to a solution of a denatured and reduced Cn5 toxin. Amazingly, all four disulfide bonds in Cn5 were reformed with a total protein yield of 87% and 100% recovery of activity [34].

Artificial chaperone-assisted refolding

This method capitalizes on the ability of chemicals such as cyclodextrin to prevent the aggregation of renatured protein when denaturants and/or detergents are removed. In a typical scheme, the denatured protein is immobilized reversibly on a solid support [35] and the denaturant removed by solvent exchange with cyclodextrin. The cyclodextrin is then removed by another solvent exchange and the properly folded protein released from the column. In a variation of this strategy, Li *et al.* [36] used an immobilized cyclodextrin column to adsorb denatured *Staphylococcus aureus* elongation factor G in the presence of detergent. The detergent was removed by a simple wash and the protein removed from the column by elution with a soluble cyclodextrin. Other approaches have used immobilized cyclodextrin without detergents to refold lysozyme, carbonic anhydrase [37] or α-glucosidase [38].

Non-chromatographic procedures

Non-chromatographic procedures for protein refolding are based mainly on the rapid dilution of denatured protein in a denaturant-free solution to prevent protein aggregation. The non-aggregated species are captured by ion-exchange chromatography and are cleaned up by other chromatographic procedures. Examples include true chaperone-assisted refolding [39–43] and artificial chaperone-assisted refolding [44–48]. Both procedures are generally of low yield, prone to protein aggregation, and not very efficient in terms of chaperone reuse.

In an interesting application of refolding by dilution, an increasing number of biologically active multiprotein complexes of mammalian immune proteins, normally considered unsuitable for expression in bacterial systems owing to the lack of complicated mammalian assembly machinery have been produced with suitable quality for structural studies. The rapid dilution strategies work surprisingly well for refolding protein complexes of major histocompatibility complexes class II or class I molecules, alone or in the presence of captured peptide [49–51]. Protease mutants [52] have also been successfully refolded using this approach. The disulfide bonds are typically formed using a mixture of reduced/oxidized

glutathione, and glycerol, L-arginine or even low concentrations of urea (2–4 M) are added to prevent protein aggregation during refolding.

The refolding of active proteins can also be accomplished using high pressure. This method was successfully applied to obtain functional nuclear receptor proteins, such as farnesoid X receptor, estrogen receptor β, and liver receptor homolog 1 LRH1 [53•]. Purified inclusion bodies were sealed in a bag and placed in a pressure cell that was slowly pressurized to 2.5 kbar. After a 16 h incubation at room temperature, the cell was slowly depressurized and soluble proteins were purified by SEC. All three receptors were biologically active, as shown by binding to their putative ligands.

The refolding of proteins in free solution is most commonly used owing to its simplicity, low cost and surprising effectiveness. The method is becoming more popular as random refolding screening kits are offered commercially for different protein targets, including transmembrane proteins (EMD Biosciences, Inc., San Diego, CA). These kits can be routinely used to screen for proper refolding conditions before using a fully automated (e.g. AKTAExpress; GE Healthcare, Piscataway, NJ) protein purification system. The AKTAExpress system is optimized for purifying soluble proteins in up to four steps: affinity chromatography (His-tag or GST-tag), desalting, ion exchange, and gel filtration. The refolding of proteins is generally not recommended with the supplied low-pressure (0.3 MPa pressure limit) columns, owing to protein aggregation during purification. Instead, higher pressure resins and batch packing of the columns are preferred.

Conclusions

This review describes principal recombinant protein expression systems and summarizes their main features. The most commonly used system, prokaryotic expression, typically delivers the highest protein yield per volume of culture, but can result in frequent aggregation of the expressed proteins. The current state-of-the-art methods for converting aggregated proteins into soluble and biologically active species can be divided into two groups: those using chromatography and those using refolding in solution as the main method of conversion. The first group usually gives better quality proteins and higher yield, but requires expensive hardware. The second group does not require a high setup cost and is most commonly used, but the quality of the proteins obtained and the overall protein yield are typically lower. Chromatography methods use three major strategies to remove denaturant and to refold proteins: SEC with free protein in solution, a linear gradient while the protein is reversibly immobilized to the solid support, and chaperone-assisted refolding. These are typically the methods of choice when dealing with multiple-disulfide, difficult-to-refold proteins. The free-solution methods typically use refolding by dilution into a denaturant-free solution, with or without natural (GroEL or its fragments) or artificial (cyclodextrin or its derivatives) chaperones. More recently, high pressure methods have also been used to solubilize inclusion bodies. These solution-based methods are optimal for assembling protein complexes and can be easily automated for the random screening of refolding conditions both for soluble and transmembrane proteins. This screening feature can be easily included in the choice of final solvent when refolding or purifying proteins by automated chromatographic purification systems.

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